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Naturally Occurring Biofilms on Alfalfa and Other Types of Sprouts†

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ABSTRACT

Scanning electron microscopy was used to examine the cotyledons, hypocotyls, and roots of alfalfa, broccoli, clover, and sunflower sprouts purchased from retail outlets as well as alfalfa sprouts grown in the laboratory using a tray system equipped with automatic irrigation. Biofilms were observed on all plant parts of the four types of commercially grown sprouts. Biofilms were also commonly observed on alfalfa sprouts grown in the laboratory by 2 days of growth. Rod-shaped bacteria of various sizes were predominant on all sprouts examined both as free-living cells and as components of biofilms. Occasionally, cocci-shaped bacteria as well as yeast cells were also present in biofilms. The microbes contained in the biofilms appeared to be attached to each other and to the plant surface by a matrix, most likely composed of bacterial exopolysaccharides. Biofilms were most abundant and of the largest dimensions on cotyledons, sometimes covering close to the entire cotyledon surface (approximately 2 mm in length). Naturally occurring biofilms on sprouts may afford protected colonization sites for human pathogens such as *Salmonella* and *Escherichia coli* O157:H7, making their eradication with antimicrobial compounds difficult.

In natural and industrial environments, bacteria are commonly found not as free-living cells but rather as adherent cells present in the form of biofilms *(1)*. Mature biofilms are structured communities of microbes adherent to a surface (usually inert) and embedded in self-produced glycocalyx material (primarily exopolysaccharides) *(2)*. Biofilms are currently the subject of intense interest due to their widespread occurrence in nature and their importance in clinical and industrial settings *(2, 8)*. The process of biofilm formation is believed to start with adhesion of individual microbes to a surface, aggregation into microcolonies, intercellular communication through quorum sensing signals, and finally maturation into structured biofilms *(2)*.

Bacteria present in biofilms differ greatly from their free-living counterparts that are commonly studied in the laboratory. For example, biofilm bacteria are 500 times or more resistant to antimicrobial compounds, are derepressed for a large number of genes, and interact physiologically with other microbes as a complex community *(1)*. Biofilms have been commonly observed on inert surfaces in aquatic systems, on surfaces of medical devices, and in industrial settings including food contact surfaces. In contrast, there have been relatively few reports of biofilms on the surfaces of living plant or animal tissues. The opportunistic pathogen *Pseudomonas aeruginosa* forms biofilms in the lungs of cystic fibrosis patients *(2)*, and recently, biofilms have been described on the surface of leafy vegetables such as lettuce and spinach *(9, 10, 13)* as well as pasture grass *(15)*.

Since 1995, there have been numerous outbreaks of foodborne disease both in the United States and worldwide due to contaminated sprouts. Most of these outbreaks were due to contamination of alfalfa sprouts with various *Salmonella* serotypes *(12, 17)*. Radish- and alfalfa sprout-related outbreaks due to contamination with *Escherichia coli* O157 have also occurred. Sprouts represent a unique challenge to growers in that during the germination and growth process environmental conditions are highly conducive to rapid bacterial growth and spread. Sprouts are normally grown at room temperature in trays or rotary drums equipped with automatic spray irrigation systems so that the sprout surfaces are continuously wet during the growth period (usually 4 to 7 days). Thus, contamination of seed with low levels of a human pathogen can result in a final product that supports high populations of the pathogen.

Research is underway in several laboratories including our own on the development of effective seed decontamination techniques because the majority of sprout-related disease outbreaks appear to be due to the use of contaminated seed. There is also interest in adding antimicrobial compounds to the irrigation water to inhibit the growth of any human pathogens introduced after seed decontamination due to inadequate water quality or insufficient worker hygiene. Studies in our laboratory have indicated that it is very difficult to achieve a significant reduction in the natural microflora of growing sprouts by addition of antimicrobials to the irrigation water *(4)*. In light of these findings we hypothesized that the natural microflora was present, at least in part, in the form of biofilms. To prove or disprove

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this hypothesis, commercially produced sprouts, as well as sprouts grown in our laboratory, were examined by scanning electron microscopy (SEM). The results of these studies support our hypothesis and are reported here.

MATERIALS AND METHODS

Sprouts. Alfalfa, broccoli, clover, and sunflower sprouts were purchased from retail outlets. Sprouts that appeared fully turgid and free of any fungal or bacterial rots were selected. Alfalfa sprouts were also grown in the laboratory using a tray system. Alfalfa seed (lot 6NM-249) was obtained from Caudill Seed (Louisville, Ky.). Before placing in the germination trays, seed (30 g) was surface disinfested by submerging in a solution of buffered calcium hypochlorite (65% available chlorine, Fisher Scientific, Pittsburgh, Pa.) (3 g/100 ml of 500 mM potassium phosphate buffer, pH 6.8) for 10 min. The treated seed was rinsed twice with 200 ml of sterile tap water. The rinsed seed was left to soak in sterile tap water for 2 h, placed into trays, and the trays put into the sprouting apparatus. The seed and resultant sprouts were watered automatically with tap water for 1 min at 30-min intervals for 4 days. For SEM observations, samples of the growing sprouts were removed daily.

To determine if rinsing sprouts would remove the biofilms, 4-day-old alfalfa sprouts grown in the laboratory were harvested, and a sample of sprouts without seed coats still attached to the cotyledons were held under running tap water for 2 min. After rinsing, the sprouts were gently blotted between paper towels. Rinsed and unrinsed 4-day-old alfalfa sprouts were then processed for SEM.

SEM. Sprouts were immersed in 2% glutaraldehyde prepared in 0.1 M imidizole-HCl buffer, pH 7.0, left for 2 h at room temperature, and then stored at 4°C. For further processing, samples were washed twice with buffer alone, immersed in 2% osmium tetroxide prepared in buffer, washed with distilled water, dehydrated in a graded series of ethanol solutions, and then critical point dried from liquid carbon dioxide. The dried samples were mounted on aluminum specimen stubs with colloidal silver adhesive paste (Electron Microscopy Sciences, Fort Washington, Pa.) and coated with a thin layer of gold in a model LV76 sputtering unit (Plasma Sciences, Lorton, Va.). If the sprouts were too large to mount whole on the aluminum specimen stubs, they were first cut with a stainless steel razor blade into cotyledons, hypocotyls, and roots. At least four sprouts of each type (or for each time period in the case of laboratory-grown alfalfa sprouts) were examined. Digital images were collected using an Imix-1 digital imaging workstation (Princeton Gamma-Tech, Princeton, N.J.) integrated with a model 840A scanning electron microscope (JEOL USA, Peabody, Mass.) operated in the secondary electron imaging mode.

Enumeration of natural bacterial populations on alfalfa sprouts. Alfalfa seed (90 g) (lot 6NM-249) was surface disinfested as outlined above. After the 2-h soak period, the seed was placed in seed trays in the sprouting apparatus for a total of 4 days. On days 1 through 4, sprouts were removed from the trays and blotted between sterile paper towels. For each day, half of the sample (10 g) was placed in a sterile stomacher bag along with 40 ml of 100 mM potassium phosphate buffer, pH 6.8. The bag contents were gently massaged by hand for 2 min. The other 10-g sample was added to a sterile stainless steel blender receptacle containing 40 ml of the potassium phosphate buffer and subjected to homogenization with a commercial blender for 30 s. One-milliliter samples were removed from the stomacher bags or from the blender receptacle and decimal dilutions were prepared using 0.1% peptone–water as diluent. Both undiluted and diluted samples (1.0 ml) were plated in duplicate onto 3M (St. Paul, Minn.) Total Aerobes Petrifilm plates. The inoculated Petrifilm plates were incubated at 30°C for 48 h, and the bacterial colonies were counted. The experiment was done two times.

RESULTS

The surfaces of alfalfa, broccoli, clover, and sunflower sprouts purchased at local retail outlets were examined by SEM for the presence of naturally occurring biofilms. For the purpose of this study, biofilms were defined as tightly packed assemblages of microbial cells totally or partially covered by matrix material that appeared to bind the microbes to each other and to the plant surface. Biofilms were observed on all four types of commercially grown sprouts, on every sprout examined, and on all three types of plant surfaces (cotyledons, hypocotyls, and roots). They were most easily located on cotyledon surfaces (Figs. 1A and B, 2A and B, 3A and B, and 4B) followed by the surfaces of hypocotyls (Figs. 1C, 3C, and 4A), and least often observed on the surface of roots (Figs. 1D, 2C and D, 3D, and 4C). Rod-shaped bacteria of various sizes were the predominant microbes observed on all surfaces of all types of sprouts examined. Cocci-shaped bacteria (Fig. 2B) as well as yeast (Fig. 3A) were observed only rarely, while structures resembling filamentous fungi were not observed.

To determine how early in the propagation process microcolonies and biofilms are formed, alfalfa sprouts were grown in the laboratory using a tray system equipped with automatic irrigation. Samples of sprouts at days 1 through 4 of propagation were harvested and processed for viewing by SEM. Four sprouts for each time period were examined. Biofilms were first observed at day 2 on the hypocotyl surfaces (Fig. 4A). At days 1 and 2, cotyledons were still fully or mostly covered by remnants of the seed coats. Biofilms were observed on both hypocotyl and cotyledon (Fig. 4B) surfaces on 3-day-old sprouts. At both days 3 and 4, one of the four sprouts examined had a cotyledon (2 to 3 mm in length) almost completely covered by a single, continuous biofilm (not shown). Biofilms were first located on roots at day 4 (Fig. 4C). Biofilms were easily located on 4-day-old alfalfa sprouts that were rinsed under running tap water.

In an attempt to estimate what percentage of bacteria (total mesophyllic aerobes) were present in the form of biofilms on laboratory-grown alfalfa sprouts, samples of growing sprouts were harvested. The harvested sprouts were divided in half, and populations were quantified by two methods. In the first method, sprouts suspended in sterile buffer were gently hand massaged for 2 min followed by plating. This method should suspend loosely or nonadherent bacteria but would not be expected to remove bacteria that are firmly attached to the plant surface as members of biofilms. The second method consisted of homogenization of the plant tissues followed by plating. This method should release the majority of bacteria from the plant surface including those present in biofilms. Thus, the difference in microbial populations determined by the two methods may

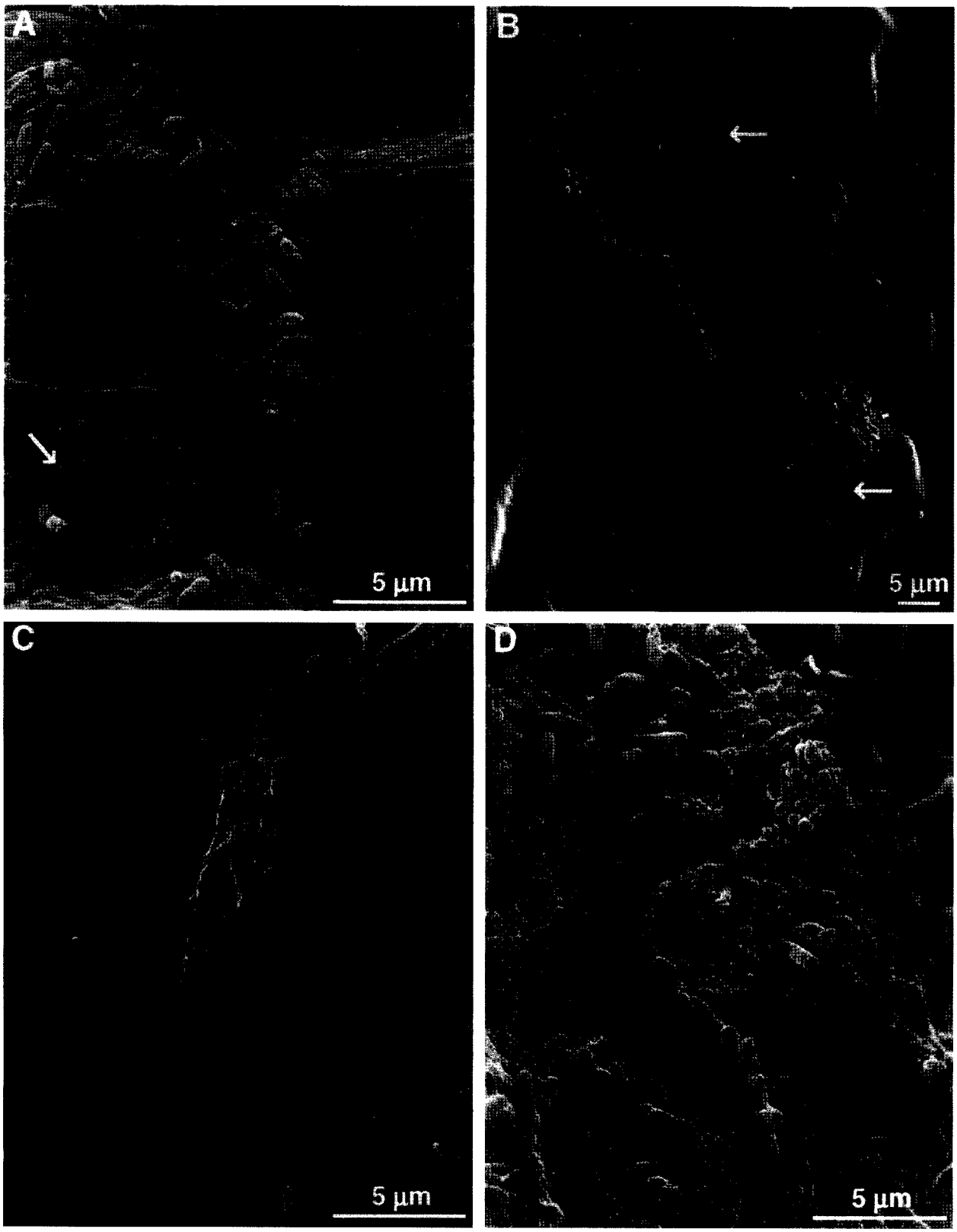

FIGURE 1. *Scanning electron micrographs of commercially grown alfalfa sprouts. (A) An incipient biofilm on the surface of a cotyledon, note the strands (arrow) of material attaching the bacteria to each other and to the plant surface; (B) two biofilms (arrows) on a cotyledon surface, note the almost continuous matrix of material between and covering the bacterial cells; (C) a biofilm on the surface of a hypocotyl, note the mixed bacterial cell morphologies; (D) a biofilm on the surface of a primary root.*

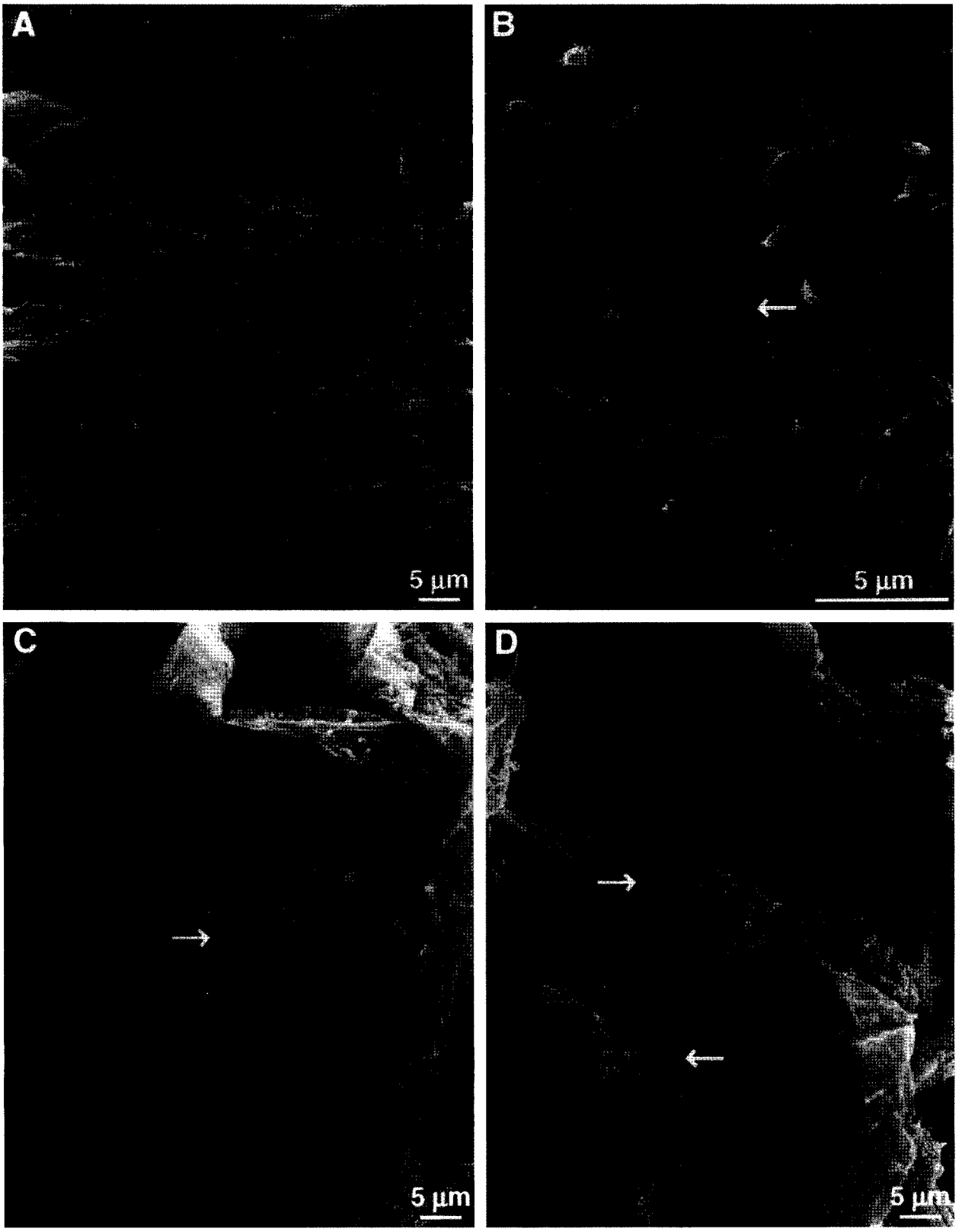

FIGURE 2. *Scanning electron micrographs of biofilms on commercially grown broccoli sprouts. (A, B) Biofilms on cotyledon surfaces, note the presence of cocci-shaped cells (arrow) (B); (C, D) biofilms (arrows) on secondary root surfaces.*

provide an estimate of the microbial populations present in biofilms at the different time periods. Results are shown in Figure 5. The data indicate that the percentage of the total mesophyllic bacteria present in biofilms ranged from 29 to 59% over the 4-day period.

DISCUSSION

The presence of natural biofilms on inert surfaces, especially in flowing aquatic systems, was observed many years ago *(1)*. However, the occurrence of natural biofilms

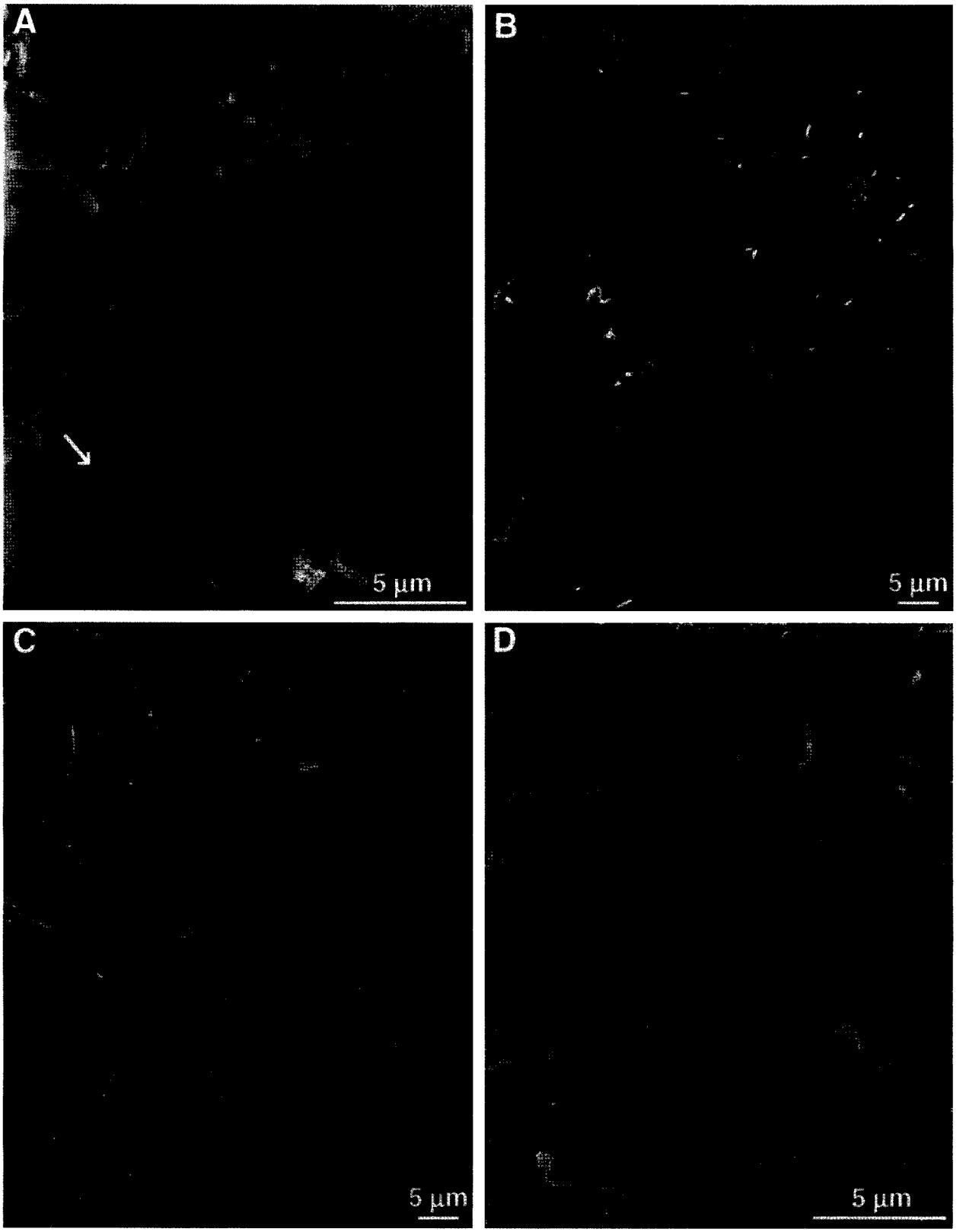

FIGURE 3. *Scanning electron micrographs of biofilms on commercially grown clover (A) and sunflower (B, C, D). (A, B) Biofilms on cotyledon surfaces, note the presence of yeast (arrow) A; (C) a biofilm on the surface of a hypocotyl; (D) a biofilm on the surface of a primary root.*

on the surfaces of plants has only recently been reported *(5, 9, 10, 15)*. By use of epifluorescence microscopy, SEM, and confocal scanning laser microscopy, Morris and associates *(9, 10)* demonstrated the presence of naturally occurring biofilms on the surface of a variety of commercially produced leafy vegetables such as spinach, lettuce, broadleaf endive, and parsley. Most of the biofilms on the leaf surfaces were associated with trichomes (leaf hairs) and

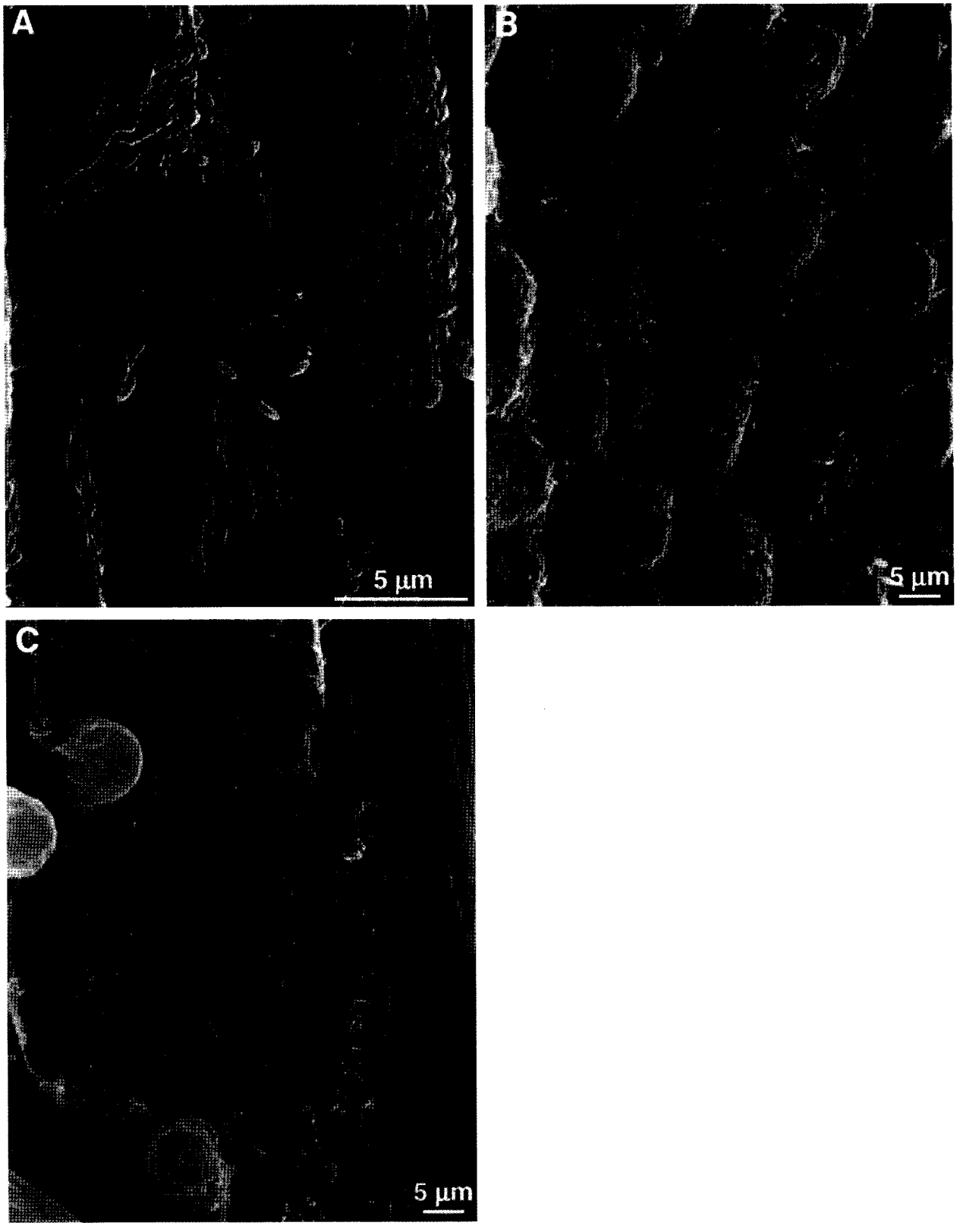

FIGURE 4. *Scanning electron micrographs of biofilms on laboratory-grown alfalfa sprouts. (A) A biofilm on the surface of a hypocotyl at day 2, (B) biofilms on the surface of a cotyledon at day 3, and (C) a biofilm on the surface of a primary root at day 4.*

were heterogeneous in nature, being composed of gram-negative and gram-positive bacteria as well as yeast and filamentous fungi *(9)*. The biofilms were up to 1 mm in length and 20 μm in depth *(9)*. Almost all of the biofilms that we observed on commercially produced alfalfa, broccoli, clover, and sunflower sprouts as well as alfalfa sprouts grown in the laboratory were composed primarily of rod-shaped bacterial cells of varying dimensions. Only one biofilm made up of predominantly cocci-shaped bacteria was noted. Yeast was rarely observed embedded in biofilms, and

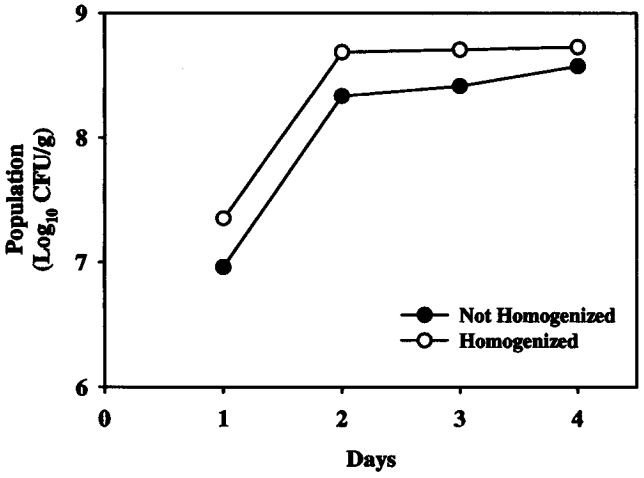

FIGURE 5. *Enumeration of total mesophyllic aerobes on laboratory-grown alfalfa sprouts. Sprouts were either agitated by hand in buffer or homogenized in buffer before enumeration of bacteria by direct plating. Values shown are the averages of data from two separate experiments.*

no biofilms containing filamentous fungi were detected. The predominance of rod-shaped bacteria that we observed on alfalfa sprouts correlates well with the findings of Splittstoesser et al. *(14)* where 96% of the isolates obtained from alfalfa sprouts were rod-shaped. The largest biofilms (up to 2 mm in length) observed were on the cotyledons of 3- and 4-day-old alfalfa sprouts grown in the laboratory. Morris et al. *(10)* estimated that bacteria in biofilms constituted between 10 and 40% of the total bacterial population on parsley and broad-leaf endive. The results of our bacterial population studies with laboratory-grown alfalfa sprouts gave similar results for the percentage of total mesophyllic aerobes present in biofilms that ranged from 29 to 59% over the 4-day period. Biofilms were most difficult to localize on root surfaces. This result may have been due, at least in part, to the highly uneven topography of the primary roots.

After observing the presence of biofilms on various types of commercially produced sprouts we determined if biofilms on sprouts were formed preharvest or only after harvest. For this purpose we grew alfalfa sprouts in the laboratory and examined their surfaces daily for up to 4 days of propagation. Biofilms were first evident on hypocotyls at day 2 and on cotyledons at day 3. At day 4, biofilms were first observed on roots. The presence of biofilms on sprouts at early stages of propagation supports our original hypothesis and makes the reduction of microbial loads on sprouts by addition of antimicrobial compounds to the irrigation water problematic. The common occurrence of natural biofilms on the surface of fruits and vegetables may account for the fact that rinsing with water usually reduces total bacterial counts only by 1 log cycle or less *(11)*.

The human pathogens *Salmonella* and *E. coli* O157:H7 are known to be able to form homogeneous biofilms on inert surfaces *(3, 7)*. The ability of these two human pathogens, both of which have been associated with disease outbreaks due to contaminated sprouts, to produce homogeneous biofilms on sprouts and other plants is not known. Taormina and Beuchat *(16)* recently tested a variety of antimicrobial sprays for their ability to eliminate or reduce *E. coli* O157:H7 on alfalfa seed and sprouts. None of the chemicals were effective, which may indicate biofilm formation on growing alfalfa sprouts by *E. coli* O157:H7. Even if human pathogens cannot produce homogeneous biofilms, they may become part of heterogeneous biofilms produced by nonpathogenic bacteria, making them much more recalcitrant to the inhibitory effects of antimicrobial compounds. However, the results of Seo and Frank *(13)* indicate that *E. coli* O157:H7 may not preferentially colonize biofilms produced by natural microflora, at least on leaves of lettuce. Also, the addition of antimicrobial compounds to irrigation water would, most likely, not be effective in killing human pathogens that are located in the internal tissues of sprouts. Recently, Itoh et al. *(6)* employed immunofluorescence and immunoelectron microscopy as well as direct isolation from surface-sterilized radish tissues to demonstrate that *E. coli* 0157:H7 can enter into the internal tissues of radish sprouts grown from seeds experimentally inoculated with this pathogen. Whether or not biofilms on the surfaces of plants can be as structurally and functionally complex as found for mature biofilms formed on inert surfaces is an important subject for further research.

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